Honors Thesis

Development of Microsatellite Primers for Investigating Genetic Connectivity and Diversity of Scleractinian Corals

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ABSTRACT

We focus on the development of microsatellite loci isolated from two scleractinian coral species (*Montastraea cavernosa* and *Porites astreoides*). To analyze the genetic structure of scleractinian coral populations, it is essential to identify a genetic marker that can evaluate individual or population-level differences (Shearer and Coffroth 2004). Optimal PCR conditions are essential when developing microsatellite loci and were determined through numerous PCRs in varying $MgCl_2$ concentrations, Taq polymerase concentrations, annealing temperatures, and the number of PCR cycles. Sequencing confirmed the presence of microsatellite repeats only in loci McGA5 (8 and 12 repeats) and McCA9 (5, 6, and 8 repeats). The microsatellite repeats confirmed length variability among clones within these two loci rendering them useful in population genetic analyses. Genetic analysis of population differentiation and measurements of gene flow provides a powerful tool for investigating dispersal. The creation of two more microsatellite primers will add to the available resources for analyzing the genetic connectivity and diversity of this scleractinian coral species.

INTRODUCTION

Coral reefs are considered to be one of the most biologically diverse marine ecosystems on earth and are often called the "rainforests of the sea" (Knowlton 2001). A stable marine environment suitable for the maintenance of biodiversity can be maintained by coral reefs. Coral reefs are distributed in patches along shelves that range from 2-130 ft below the surface and are presumably connected because of movement through ocean currents. When coral populations are dependent on outside source populations, it is less likely that the populations will become extinct with the presence of disease or local bleaching. On the other hand, a coral population

that is dependent on local adults suffers a barrier to population replenishment if disease is present, and this barrier can cause local extinction (Kojis and Quinn 2001). Physical impacts including overfishing, anchor damage, pollution, and the development of coastal areas can damage and threaten corals. Damage can also result from coral bleaching due to the exceptionally high water temperatures that have been reported throughout the Caribbean since the early 20th century. To prevent further coral damage, we need to maintain genetic diversity. Coral reefs rich in biodiversity are stronger and can withstand environmental stressors compared to those not rich in biodiversity (Petit et al. 1998).

The two coral species of focus in this study are from the Cnidarian family Scleractinia: *Montastraea cavernosa* and *Porites astreoides*. Scleractinian corals receive their nutrient and energy resources in two necessary ways. First, they use the traditional cnidarian strategy of capturing tiny planktonic organisms with their nematocyst-capped tentacles. Secondly, they have an obligate symbiotic relationship with zooxanthellae, which provide the corals energy via photosynthetic waste products. Sessile marine organisms, such as corals, depend on water to bring their gametes together. Corals themselves are commonly hermaphroditic and exhibit two primary types of reproductive modes: broadcast spawning and brooding (Knowlton 2001). In broadcast spawning, larval fertilization and development occur outside the maternal colony in the water column. This can then result in widespread dispersal of larvae since larvae travel with water currents during development. Brooding corals exhibit a very different reproductive strategy. Only sperm are released, while eggs are fertilized within the maternal colony where larvae develop and are eventually released as swimming larvae (Knowlton 2001). In this mode, the larvae are capable of settling on nearby substrate shortly after release from the maternal colony potentially resulting in limited larval dispersal. Thus, sperm from one colony need to

reach eggs from another for reproduction to succeed. Therefore, reef degradation may lead to sharply reduced reproduction, not only because of lowered gamete production, but also because of reduced rates of fertilization for those gametes that are released.

The development of appropriate conservation and management strategies is dependent on the amount of information available on population connectivity and the identification of larval sources (Palumbi 2003). Currently though, local levels of connectivity and genetic diversity of coral populations are mainly unknown (Lee et al. 1994). There is an increasing need to study recent levels of gene flow among marine populations since decreasing coral cover and changes in coral species composition due to natural and human disturbances have become common among Caribbean reefs (Hughes and Tanner 2000). The first question to be addressed is, what are the fine-scale levels of genetic structure of coral species? Secondly, what is the level of gene flow among populations mediated via larval dispersal? What is the genetic diversity of populations of these species?

Montastraea cavernosa is a gonochoric (not hermaphroditic) broadcast spawning species whereas *Porites astreoides* is a hermaphroditic brooding species. This project focuses on the development of microsatellite loci isolated from two scleractinian coral species (*Montastraea cavernosa* and *Porites astreoides*).

Microsatellites sample a large portion of total genomic variation. Identifying a genetic marker that is able to evaluate individual or population-level genetic differences is essential for analyzing the genetic structure of scleractinian coral populations (Shearer and Coffroth 2004). Microsatellites are loci that vary in the number of repeats of a simple DNA sequence, and are commonly used in the analysis of natural populations (Slatkin 1995). Microsatellite markers have proven useful for population genetic analysis in numerous organisms due to their high

degree of polymorphism (Schlotterer 2000, Sunnucks 2000; Baums et al. 2005). The fact that microsatellites are highly polymorphic makes them ideal for analyzing the number of migrants between populations (Gaggiotti et al. 1999).

Microsatellites prove useful for assessing population structure and determining the relationships among very closely related species (Goldstein et al. 1995). Access to microsatellites for organisms without sequence information requires direct characterization, through the phases of cloning, detection of microsatellites and sequencing, in order to determine flanking sequences that can then be used for defining locus-specific PCR primers (Queller et al. 1993; Jarne & Lagoda 1996). Use of these markers will establish patterns of genetic variation among local and regional populations, deduce gene flow among populations, quantify genetic connectivity among reefs and identify sources of larval recruits (Shearer and Coffroth 2004).

Defining the scale of connectivity, or exchange, among marine populations and determining the factors driving this exchange are crucial to our understanding of the population dynamics and genetic structure of many coastal species (Cowen et al. 2006). It has proved difficult to either measure the frequency with which long distance movements during the larval phase occur, or alternatively to identify dispersal barriers that may act to isolate populations over ecological or evolutionary time. Data on ecological connectivity is essential while gene flow over evolutionary time scales will determine genetic structure of biodiversity in marine ecosystems (Thorrold 2006). Current research has lead to population models that try to describe the population structure of Caribbean corals (Galindo et al. 2006). Reports of population differentiation within the Caribbean have been accumulating. Further research needs to be done in order to provide a more accurate representation. To provide a more accurate model, the movement of larvae needs to be better understood.

The movements of larvae among marine populations are difficult to follow directly and have been the subject of much controversy, especially in the Caribbean. The debate focuses on the degree to how much populations are "demographically open". The questions are whether depleted populations can be replenished from distantly healthy populations or whether they are "demographically closed" and in need of conservation efforts (Baums et al. 2005b). Microsatellite loci have been isolated from the Caribbean coral *Montastraea annularis*, and this will be useful when assessing gene flow patterns and diversity of this coral species as well as understanding the population connectivity within the Caribbean (Severance et al. 2004). In a study performed by Iliana Baums and her group, five microsatellite markers were developed and demonstrated to be both Mendelian and coral-specific. Using these markers, Baums' group was able to show that populations of the Caribbean coral, *Acropora palmata*, have experienced little to no recent genetic exchange between the western and the eastern Caribbean (Baums et. al 2005).

High connectivity between populations would have important implications for the management of marine resources. For example, a smaller number of marine reserves would theoretically be required to achieve adequate protection of larval supplies, whereas highly structured populations would require a larger number of reserves (Baums et al. 2005b). Cowen et al (2000) suggested that high diffusion and mortality rates, assisted by behavioral adaptations, should result in local larval retention and closed populations over ecologically relevant timescales.

It is likely that sites supplied abundantly from "upstream" reef areas will be more resilient to recruitment overfishing, less susceptible to species loss, and less reliant on local management than places with little "upstream" reef. Strong connectivity among areas implies

that local populations may depend on processes occurring elsewhere (Roberts 1997). Knowledge of larval exchange among populations of marine organisms is fundamental to the study of marine population dynamics. Decisions made on the assumption that larvae are widely dispersed may lead to false actions if the assumption is wrong (Carr & Reed 1993). However, lack of evidence has generally resulted in the acceptance of the concept of well-mixed populations on both ecological and evolutionary scales (Cowen et al. 2000).

Developing microsatellites in *Montastraea cavernosa* and *Porites astreoides* can allow for the quantification of genetic connections among reefs and identify sources of larval recruits (Shearer and Coffroth 2004). This project will investigate genetic diversity and connectivity (via larval dispersal) among populations of scleractinian corals. This knowledge can then be used to determine effective ways to manage coral population size thus preserve a suitable habitat for species of fish.

The results obtained from this investigation are essential to the understanding of natural recruitment processes and diversity of coral species. A model of larval exchange for both broadcast spawning and brooding corals will be developed through the combination of fine-scale genetic structure determined in this study with levels of connectivity. We expect *Montastraea cavernosa* colonies will represent a panmictic population as a result of widespread larval dispersal capabilities because it is a broadcaster and will be well connected with adjacent reefs. We expect the genetic diversity of *Montastraea cavernosa* will be high due to larval inputs from multiple source populations. We expect *Porites astreoides* colonies will be genetically patchy as a result of larval dispersal being limited because it is a brooder and therefore will be genetically differentiated from adjacent reefs.

MATERIALS AND METHODS

Populations of *Montastraea cavernosa* and *Porites astreoides* were collected from Conch Reef in the Florida Keys. Collection sites were selected randomly at each depth (one at 30', two at 60', and one at 90'). Different collection sites were used to maximize the probability for variability. Coral tissue was scraped off the polyps for each sample, preserved in high salt preservative, and was further analyzed by DNA extraction. DNA was extracted from the coral samples using Qiagen DNA extraction kit. DNA that was extracted and purified from populations of *Montastraea cavernosa* and *Porites astreoides* were used in the development of microsatellite primers.

Species-specific microsatellite primers (McGA5, McGA7, McGA10, McGA12, McCA9, McCA16, PaCA8, PaCA28, PaCA32, PaCA43, and PaCA56) were designed from microsatellite libraries developed for *M. cavernosa* (Mc) and *P. astreoides* (Pa) (Shearer and Coffroth 2004). DNA amplification with these primers requires optimal conditions to maximize the PCR amplifications. Optimal PCR conditions were determined through numerous PCRs in varying $MgCl_2$ concentrations, Taq polymerase concentrations, annealing temperatures, and the number of PCR cycles (Table 1). Concentrations of $MgCl_2$ and primers specific to each locus were optimized following: initial denaturation at 94 ºC for 2 min. followed by 17 cycles of denaturation at 94 ºC for 15 s, annealing at a primer-specific initial temperature of 64 ºC for 15 s with a decrease of -1.0 ºC per cycle, and extension at 72 ºC for 2 min. With the new annealing temperature the program continued with 35-40 cycles of denaturation at 94 ºC for 15 s, annealing at a locus specific temperature ranging from 47 ºC to 60 ºC for 15 s and extension at 72 ºC for 2 min. The final cycle is followed by an extension cycle at 72 ºC lasting for 10 min.

To confirm the presence of microsatellite regions in the amplification product, alleles were sequenced. For each locus, putative microsatellite alleles from 10 coral colonies of each species were amplified. The DNA was combined and cloned using the TOPO Cloning Reaction and Transformation kit. The TOPO Cloning kit allows for the ligation of PCR product into the plasmid and the transformation of the recombinant vector that is competent to *E. coli* cells. Single large white colonies were picked and grown on LB plates overnight. Clones were grown and plasmids were purified with QIAprep Spin Mini Prep kit to purify the plasmid DNA for sequencing. Visualization of plasmid insertion was confirmed through PCR with M13 forward and reverse primers. Alleles for each locus were sequenced in the forward direction using M13 forward primer. DNA was submitted to Nevada Genomics for sequencing. Microsatellite alleles from the locus specific primers were sequenced using an ABI Genetic Analyzer to determine if these loci were appropriate for population genetic analyses (i.e. presence and variability of a repetitive region). Sequences for each locus were aligned to assess length polymorphism within each microsatellite locus.

RESULTS

Primers McGA5, McGA12, McCA9, PaCA8, PaCA28, and PaCA43 were optimized to show amplification with a single band. The remaining primers (PaCA56, PaCA32, McGA10, McGA7, McCA16) were eliminated due to multiple bands or no amplification product. All successful primers had greatest amplification with 1 or 2 U Taq polymerase (Table 2). Primers had the greatest amplification with a $MgCl_2$ concentration of either 2.5 mM or 3.0 mM (Table 2). Microsatellite alleles could be visualized on a 2% TBE agarose gel stained with EtBr (Figure 1 and Figure 2). Sequencing confirmed the presence of microsatellite repeats only in loci McGA5

(8 and 12 repeats) and McCA9 (5, 6, and 8 repeats) (Table 3). The microsatellite repeats confirmed length variability among clones within these two loci rendering them useful in population genetic analyses. Other loci (McGA12, PaCA8, PaCA28, and PaCA43) did not confirm microsatellite repeats. These loci were cloned, but did not display a repetitive region. Plausible explanations are that the repeats were not long enough to display length variability or that non-specific DNA was inserted during the transformation.

DISCUSSION

With the confirmation of microsatellite repeats in two *Montastraea cavernosa* loci, the microsatellite primers can now be fluorescently tagged and used to further genetically characterize our target *M. cavernosa* coral populations. Microsatellite allele data will be analyzed to determine allele frequencies, heterozygosities, and ultimately describe gene flow among populations. To investigate potential sources of larvae for populations at Conch Reef, F-statistics and Bayesian methods can also be used to determine gene flow between adult and juvenile population and among juvenile populations.

Significant genetic differentiation among populations will indicate barriers to gene flow due to factors such as different larval sources, limited larval dispersal and/or selective factors. Genetic homogenization of these populations indicates that larval exchange was sufficient in the past and is currently sufficient to prevent significant differentiation. Genetic homogenization may indicate that these populations share a larval source. Significant differentiation between adult and juvenile populations suggests that contemporary levels of gene flow are restricted between sites that natural selection influences the genetic composition of a population over time or that larval sources have changed over time. Significant differentiation among juvenile

populations across Conch Reef can indicate that multiple sources contribute to recent recruitment events or that larval dispersal is not widespread.

Understanding the causes of population differentiation is a continuing effort in evolutionary biology. The oceanic environment provides few obvious physical barriers that may prevent genetic exchange of larvae between populations (Baums et al. 2006). However, this concept has been challenged by reports of localized recruitment and strongly structured populations (Thorrold et al. 2002). In a study performed by Ayre & Hughes (2000), it was determined that for many brooding and broadcast spawning coral species, individual reefs are dependent primarily upon self-seeding for the maintenance of populations. The finding that some broadcast spawning corals are dependent primarily upon self-seeding was not expected. Their data did not support the prediction that brooding species have more restricted dispersal than broadcast spawners. Genetic markers have suggested that several coral reef species have strongly structured populations (Swearer et al. 2002). A link between evolution and ecology can be made based on the relationship between the genetic structure and the dispersal ability of organisms within a population (Palumbi 1994; Ayre & Hughes 2000). Past and present dispersal remain poorly understood for most species. Genetic analysis of population differentiation and measurements of gene flow provides a powerful tool for investigating dispersal (Ayre & Hughes 2000). The addition of two more microsatellite primers will add to the available resources for analyzing the genetic connectivity and diversity of this scleractinian coral species. Additional markers are being developed for other species in order to have a stronger pool of available information so that conservation measures are designed with the understanding of natural recruitment processes and diversity of coral species.

TABLES/FIGURES

Table 1. Range of varying PCR conditions that were tested to maximize PCR amplifications.

$MgCl_2$ concentration	1-3 mM
Taq polymerase concentration	1-2 U
Annealing temperature	47-60°C
Number of PCR cycles	35-40

Table 2. Optimal PCR reaction conditions for *Montastraea cavernosa* (Mc) and *Porites astreoides* (Pa) primers.

Optimal Conditions	Annealing Temp °C	# of cycles	Stock primer conc.	10x	dNTPs	for	rev	$MgCl_2$	Taq
McGA5	47	40	1µM	1.5 mM	0.2 mM	.015 mM	.015 mM	2.5 mM	1 U
McCA9	47	40	1µM	1.5 mM	0.2 mM	.015 mM	.015 mM	2.5 mM	1 U
McGA12	60	40	5µM	1.5 mM	0.2 mM	.015 mM	.015 mM	2.5 mM	2 U
PaCA8	49	40	1µM	1.5 mM	0.2 mM	.015 mM	.015 mM	2.5 mM	2 U
PaCA28	52	40	10µM	1.5 mM	0.2 mM	.015 mM	.015 mM	3.0 mM	2 U
PaCA43	48	40	10µM	1.5 mM	0.2 mM	.015 mM	.015 mM	3.0 mM	2 U

Table 3. Microsatellite repeats were confirmed among clones for primers McCA9 (5, 6, and 8 repeats) and McGA5 (8 and 12 repeats). The microsatellite repeats confirmed length variability among clones within each locus.

Microsatellite Sequence Repeats	
McCA9-1	CACACACACN
McCA9-3	CACACACACN
McCA9-2	CACACACACACA
McCA9-4	CACACNCACACNCACA
McGA5-1	GAGAGAGAGAGAGAGAGAGAGAGA
McGA5-3	GAGAGNGAGAGAGAGA

Figure 1. Gel showing amplification confirmation of McCA9. L represents a 100 bp ladder, 1-5 represent coral samples, and N is the negative control. The red arrow represents the location of the single band at approximately 160 bp.

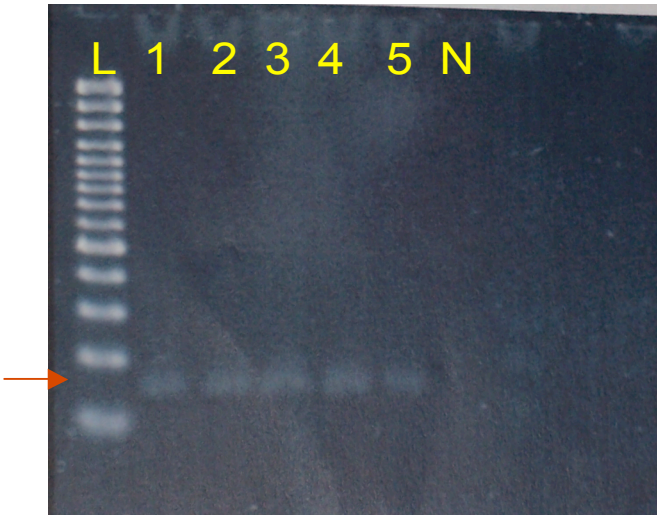
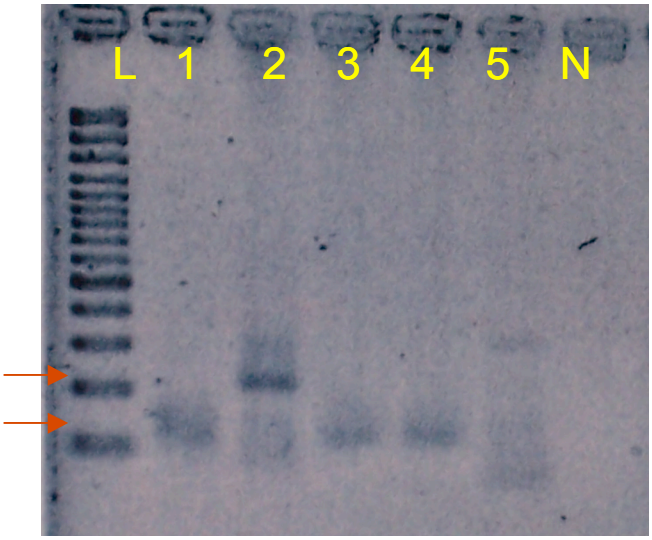


Figure 2. Gel showing amplification confirmation of PaCA8. L represents a 100 bp ladder, 1-5 represent coral samples, and N is the negative control. The red arrows represent the location of the single band at approximately either 150 bp or 200 bp due to size variability.



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